NUP214-ABL1-mediated cell proliferation in T-cell acute lymphoblastic leukemia is dependent on the LCK kinase and various interacting proteins

Kim De Keersmaecker,[1,2]* Michaël Porcu,[1,2]* Luk Cox,[1,2] Tiziana Girardi,[1,2] Roel Vandepoel,[1,2] Joyce Op de Beeck,[1,2] Olga Gielen,[1,2] Nicole Mentens,[1,2] Keiryn L. Bennett,[3] and Oliver Hantschel[4]

[1]Center for the Biology of Disease, VIB, Leuven, Belgium; [2]Center for Human Genetics, KU Leuven, Leuven, Belgium; [3]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; and [4]Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

KDK and MP contributed equally to this manuscript.

ABSTRACT

The NUP214-ABL1 fusion protein is a constitutively active protein tyrosine kinase that is found in 6% of patients with T-cell acute lymphoblastic leukemia and that promotes proliferation and survival of T-lymphoblasts. Although NUP214-ABL1 is sensitive to ABL1 kinase inhibitors, development of resistance to these compounds is a major clinical problem, underlining the need for additional drug targets in the sparsely studied NUP214-ABL1 signaling network. In this work, we identify and validate the SRC family kinase LCK as a protein whose activity is absolutely required for the proliferation and survival of T-cell acute lymphoblastic leukemia cells that depend on NUP214-ABL1 activity. These findings underscore the potential of SRC kinase inhibitors and of the dual ABL1/SRC kinase inhibitors dasatinib and bosutinib for the treatment of NUP214-ABL1-positive T-cell acute lymphoblastic leukemia. In addition, we used mass spectrometry to identify protein interaction partners of NUP214-ABL1. Our results strongly support that the signaling network of NUP214-ABL1 is distinct from that previously reported for BCR-ABL1. Moreover, we found that three NUP214-ABL1-interacting proteins, MAD2L1, NUP155, and SMC4, are strictly required for the proliferation and survival of NUP214-ABL1-positive T-cell acute lymphoblastic leukemia cells. In conclusion, this work identifies LCK, MAD2L1, NUP155 and SMC4 as four new potential drug targets in NUP214-ABL1-positive T-cell acute lymphoblastic leukemia.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of T-cell progenitors occurring in adults and children. With current chemotherapy protocols, the outcome for pediatric T-ALL has improved to cure rates of over 75%. However, these therapies are highly toxic. Moreover, adult T-ALL patients, patients with primary resistance to chemotherapy and patients who have relapsed after initial treatment have an extremely poor prognosis, underscoring the need for novel therapeutic strategies.[1,2]

T-ALL is a genetically heterogeneous disease that is caused by accumulation of multiple genetic defects in developing T cells, affecting critical cellular processes such as cell differentiation, proliferation, survival and self-renewal capacity.[3] Approximately 8% of T-ALL cases harbor fusions that involve the *ABL1* tyrosine kinase gene.[3] BCR-ABL1, which is the prototypic ABL1 fusion kinase in chronic myeloid leukemia (CML) and subsets of B-cell ALL (B-ALL), is only sporadically found in T-ALL. In contrast, 6% of T-ALL cases express the constitutively active NUP214-ABL1 fusion kinase, which consists of the N-terminal region of the nuclear pore complex protein NUP214 and of the same C-terminal part of ABL1 as in BCR-ABL1.[4] Recently, the NUP214-ABL1 fusion was also identified in B-ALL.[5] Although NUP214-ABL1 and BCR-ABL1 are both constitutively active kinases that stimulate the proliferation of the leukemic cells, they differ from each other in almost all properties we investigated, including genetic etiology, transforming capacity, substrate preference and phosphorylation pattern.[4,6-7] Furthermore, the kinase activation of NUP214-ABL1 depends on its localization at the nuclear pore complex and not on coiled-coil induced oligomerization and binding of the adaptor protein GRB2 like for BCR-ABL1.[7] Despite these differences, NUP214-ABL1 and BCR-ABL1 are both sensitive to ABL1 kinase inhibitors, and ABL1 inhibitors inhibit the proliferation of leukemic cells expressing these kinases,[4,6,8] rendering ABL1 kinase inhibitors attractive therapy for NUP214-ABL1-positive T-ALL.

Although the introduction of ABL1 kinase inhibitors was a major step forward in the treatment of BCR-ABL1-positive leukemias, it has become clear that patients treated with these inhibitors sometimes relapse due to the acquisition of resistance mutations.[9] These mutations are particularly common in BCR-ABL1-positive B-ALL and advanced CML, which, like T-ALL, are genetically more complex than chronic phase CML. Several mechanisms of resistance occur, including BCR-ABL1 mutations interfering with inhibitor binding and amplification of the *BCR-ABL1* oncogene.[10] Consequently, to find therapeu-

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Correspondence: oliver.hantschel@epfl.ch or kim.dekeersmaecker@cme.vib-kuleuven.be

tics that target therapy-resistant cells, much effort has been invested in identifying novel proteins or pathways that are required for BCR-ABL-mediated transformation.[11] An important example is the observation that certain SRC family kinases are critical signaling proteins in BCR-ABL1-positive B-ALL and advanced CML.[12-15] Indeed, the SRC family kinase LYN is over-expressed in ABL1 kinase inhibitor-resistant CML blast crisis and knockdown of LYN induces apoptosis in these cells.[14] In addition, proteins interacting with BCR-ABL1 were recently identified,[16] which may allow the identification of therapeutically relevant critical interactors. In contrast, unbiased studies of the NUP214-ABL1 signaling network are lacking and critical signaling pathways and interactors that can be therapeutically targeted may previously have been missed.

We fear that, as for BCR-ABL1, resistance to ABL1 inhibitors may develop readily in NUP214-ABL1-positive T-ALL. Although clinical experience with ABL1 inhibitors in NUP214-ABL1-positive T-ALL is limited, a recent report on a NUP214-ABL1-positive patient who had imatinib added to his therapeutic scheme did indeed show that the patient, after obtaining a rapid remission, had a fatal relapse.[17] In this study, we aimed to identify novel therapeutic targets in NUP214-ABL1-positive T-ALL by using two approaches. First, we investigated the therapeutic potential of SRC family kinases. In addition, we performed an unbiased mass spectrometry and short interfering (si)RNA-based screen to identify proteins that are critical for the survival and proliferation of NUP214-ABL1-positive T-ALL cells.

Methods

Constructs

Hemagglutinin(HA)-tagged NUP155 and SMC4 cDNAs were synthesized (Genscript) and cloned into the XhoI and EcoRI restriction sites of pMSCV-puro (Clontech). NUP214-ABL1 and BCR-ABL1 constructs are described elsewhere.[7]

Cell culture

ALL-SIL, K-562, KE-37, RPMI-8402, JURKAT, SUP-T1, RLD-1 and L-5178-Y cell lines (obtained from DSMZ) were cultured in RPMI-1640 medium with 20% fetal calf serum (FCS). NA10073 and NA10075 cell lines were established from mouse T-ALL leukemias induced in a NUP214-ABL1 bone marrow transplant assay[7] and were cultured in RPMI-1640 with 20% FCS. HEK293T cells (from DSMZ) were cultured in RPMI-1640 with 10% FCS. Transfections were performed using Turbofect reagent (Fermentas).

Immunoblotting

Samples were processed according to standard procedures using the following antibodies: anti-FYN (Fyn3), anti-LCK (3A5), anti-ABL1 (24-11) and anti-ERK2 (Santa Cruz Biotechnology); anti-NUP155 (ab73292) (Abcam); anti-phospho-SRC family (Tyr416), anti-phospho-ABL1 (Tyr245), anti-SMC4 (D14E2) and anti-MAD2L1 (D8A7) (Cell Signaling); anti-HA tag (12CA5) (Roche) and peroxidase-labeled anti-mouse/anti-rabbit antibodies (Amersham).

Short interfering RNA knockdown

Human T-ALL cell lines were electroporated on a Genepulser Xcell instrument (Biorad) with 400 nM siRNA. For mouse T-ALL cell lines, two electroporations were performed with a 24-h interval. Viable cell numbers and viability were determined on a Vi-cell XR cell viability analyzer (Beckman Coulter) or on a Guava easyCyte HT (Millipore). Knock-down efficiencies were evaluated by immunoblotting or by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. siRNA sequences are presented in *Online Supplementary Table S1*.

Quantitative reverse transcription polymerase chain reactions

qRT-PCRs were performed on a LightCycler 480 instrument (Roche). Relative expression levels were calculated according to the ΔΔCt method using *GAPDH*, *UBC* or *HPRT* as normalizer genes.

Drug sensitivity

To construct the dose-response curves, $5x10^5$ cells were seeded in 1 mL of growth medium and incubated in the presence of PP2 (Calbiochem) for 48 h. Cell proliferation was determined using CellTiter 96 AQueous One reagent (Promega). Apoptosis was analyzed using the PE Annexin V apoptosis detection kit I (BD Biosciences). Flow cytometry was performed on a FACSCanto (BD Biosciences).

Mass spectrometric analysis of NUP214-ABL1 protein complexes

Biological duplicates of the pull-down experiments and mass spectrometric analyses were described previously.[16]

(Co-)immunoprecipitation

Cells were lysed on ice for 30 mins in cell lysis buffer (Cell Signaling) supplemented with 5 mM NA_3VO_4 and complete protease inhibitor (Roche). For PP2 pretreatment, cells were incubated with PP2 for 20 h prior to lysis. Lysates were pre-cleared with protein G Dynabeads (Invitrogen) followed by overnight incubation with antibody-coupled protein G Dynabeads. The antibodies used were: anti-ABL1 (24-11) (Santa Cruz Biotechnology), anti-MAD2L1 (D8A7) (Cell Signaling) and anti-phospho-tyrosine (4G10) (Millipore).

Immunofluorescence

Transfected wells were transferred to poly-L-lysine coated cover slips. The next day, cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. Cells were stained using the following antibodies: anti-ABL1 (clone 8E9) (BD Biosciences), anti-SMC4 (D14E2) (Cell Signaling), Alexa Fluor 488 conjugated anti-mouse IgG, and Alexa Fluor 555 conjugated anti-rabbit IgG (Invitrogen). Cells were examined on a Leica TCS SP5 II (Leica Microsystems) confocal microscope. Images were analyzed in ImageJ.

Results

SRC family kinases can be activated by BCR-ABL1 and are therapeutic targets in imatinib-resistant CML.[14] We, therefore, wanted to investigate whether SRC family kinases could also play an essential role in supporting proliferation and survival of NUP214-ABL1-positive T-ALL cells. We treated the human NUP214-ABL1-positive T-ALL cell line ALL-SIL as well as the NUP214-ABL1-negative T-ALL cell lines JURKAT and SUP-T1 with increasing concentrations of the SRC family kinase inhibitor PP2. Proliferation of ALL-SIL cells was completely inhibited in the presence of 5 μM PP2. However, 5 μM of PP2 caused no or a very minor effect on the proliferation of the NUP214-ABL1-negative control cell lines (Figure 1A). Likewise, PP2 had no significant

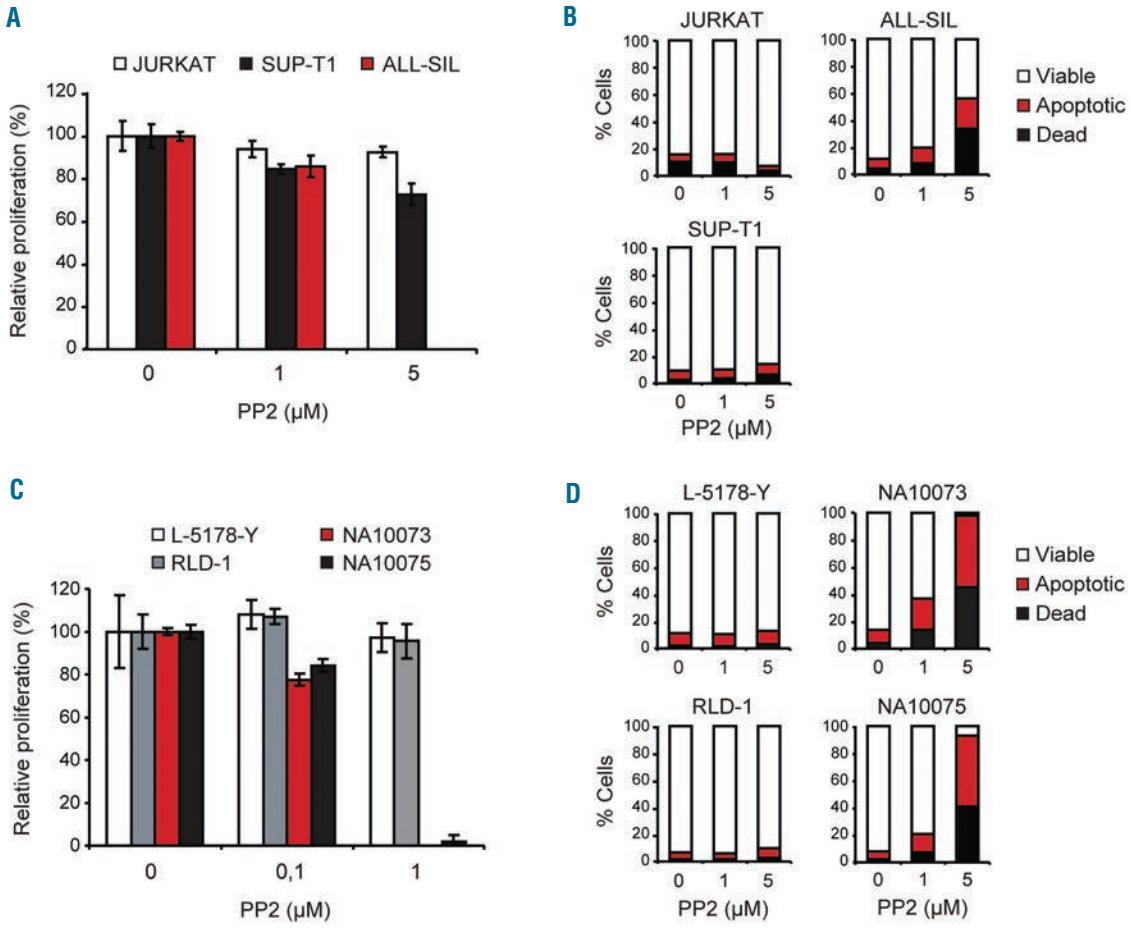

Figure 1. NUP214-ABL1-positive T-ALL cell lines are sensitive to inhibition of SRC kinases. (A) Human NUP214-ABL1-positive (ALL-SIL) and negative (JURKAT and SUP-T1) T-ALL cell lines were treated with indicated concentrations of PP2. Cell proliferation was normalized to that of DMSO-treated cells. The average ±SD of three repeats is shown. (B) Percentages of viable, apoptotic, and dead cells after PP2 treatment were determined by annexin-V/7-AAD staining. (C) Mouse NUP214-ABL1-positive (NA10073 and NA10075) and negative (L5178Y and RLD1) T-cell lines were treated with indicated concentrations of PP2 for 48 hours. Cell proliferation was normalized to that of DMSO-treated cells. The average ±SD of three repeats is shown. (D) Percentages of viable, apoptotic, and dead cells after PP2 treatment were determined by annexin-V/7-AAD staining.

effects on cell viability and apoptosis in these cells (Figure 1B). In contrast, a clear dose-dependent increase in the amount of apoptotic and dead cells was observed in response to PP2 in the NUP214-ABL1-positive ALL-SIL cells (Figure 1B). These results suggest that ALL-SIL cells depend on the activity of SRC family kinases. To verify that this effect was not restricted to the single NUP214-ABL1-positive ALL-SIL cell line, we also tested the effect of SRC family kinase inhibition in a collection of mouse T-cell leukemia cell lines. In agreement with the data obtained in the human cell lines, PP2 inhibited proliferation and induced apoptosis in the NUP214-ABL1-positive mouse cell lines NA10073 and NA10075, whereas the NUP214-ABL1 negative lines L-5178-Y and RLD-1 were unaffected (Figure 1C-1D). Notably, the effect of SRC family kinase inhibition was stronger in the mouse lines, in which a concentration of 1 μM PP2 already induced a drastic reduction of cell proliferation and survival.

To delineate the identity of the SRC family kinase(s) responsible for the sensitivity of ALL-SIL cells to PP2, we investigated the expression of eight SRC kinase family members in these cells by qRT-PCR. ALL-SIL only expressed significant levels of *LCK* and *FYN* (Figure 2A). In agreement with this, LCK and FYN proteins were detectable in ALL-SIL cells (Figure 2B). However, when we assessed the activation state of LCK and FYN in ALL-SIL cells by testing phosphorylation on their activation loops, we found that only LCK was robustly phosphorylated and that this phosphorylation was inhibited by PP2 in a dose-dependent manner. In contrast, phosphorylation of the activation loop of FYN was virtually undetectable, even in the absence of PP2 (Figure 2B). These observations indicate that only LCK kinase activity contributed to survival and proliferation of ALL-SIL cells.

As an alternative way to perturb SRC family kinase signaling, we performed siRNA knock-down experiments of LCK and FYN in ALL-SIL cells. As a positive control, we also knocked-down ABL1, which drastically interfered with the proliferation and viability of the NUP214-ABL1-dependent ALL-SIL cells (Figure 2C,D). Knock-down of LCK strongly inhibited ALL-SIL cell proliferation and viability, although to a lesser extent than knock-down of ABL1 (Figure 2C,D). This may be explained by the lower knock-down efficiencies that could be achieved for LCK as compared to ABL1 (*Online Supplementary Figure S1*). In contrast, knock-down of LCK in NUP214-ABL1-negative JURKAT

cells did not affect proliferation and viability of these cells, suggesting that dependence on LCK was specific to NUP214-ABL1-expressing cells (Figure 2C,D). Treatment with FYN siRNA induced only a slight but significant reduction in the proliferation of ALL-SIL cells but did not affect their viability. We also tested the effects of Lck knock-down in mouse T-cell leukemia cell lines, using an independent mouse Lck siRNA. In agreement with the data in the human cell lines, knock-down of Lck reduced the proliferation and survival of the NUP214-ABL1-positive cell line NA10075, whereas these effects were not observed in the NUP214-ABL1-negative cell line L-5178-Y (Figure 2E,F). Unfortunately, the NA10073 and RLD-1 mouse cell lines that we used for the experiments shown in Figure 1C,D could not be included as adequate siRNA knock-down in these cells could not be obtained. Taken together, our results indicate that NUP214-ABL1-positive human and mouse cells strongly depend on the expression and activity of LCK for their proliferation and survival and that therapeutic inhibition of LCK activity may provide an alternative means of treating NUP214-ABL1-positive T-ALL.

We next set out to identify proteins, in addition to LCK, that are required for the proliferation and survival of NUP214-ABL1-expressing cells and could possibly be exploited for therapeutic targeting. We used an unbiased approach to study the composition of cellular NUP214-ABL1 complexes by mass spectrometry-based interaction proteomics. For this, NUP214-ABL1 (along with ABL1) and its interacting proteins were immunoprecipitated with an anti-ABL1 antibody from ALL-SIL cells followed by mass spectrometric analysis of proteins in the precipitated complexes (Figure 3A). The mass spectrometry results were searched against the human International Protein Index database[18] to yield a primary dataset of 289 proteins (*Online Supplementary Table S2*). From this list, nine potential specific NUP214-ABL1 interactors were selected (Table 1). This was achieved by comparing the proteins identified in the immunoprecipitated samples with the most abundant proteins identified from total cell lysates (*i.e.* 'core' proteomes)[19] and removing ABL1 interactors. This method was previous-

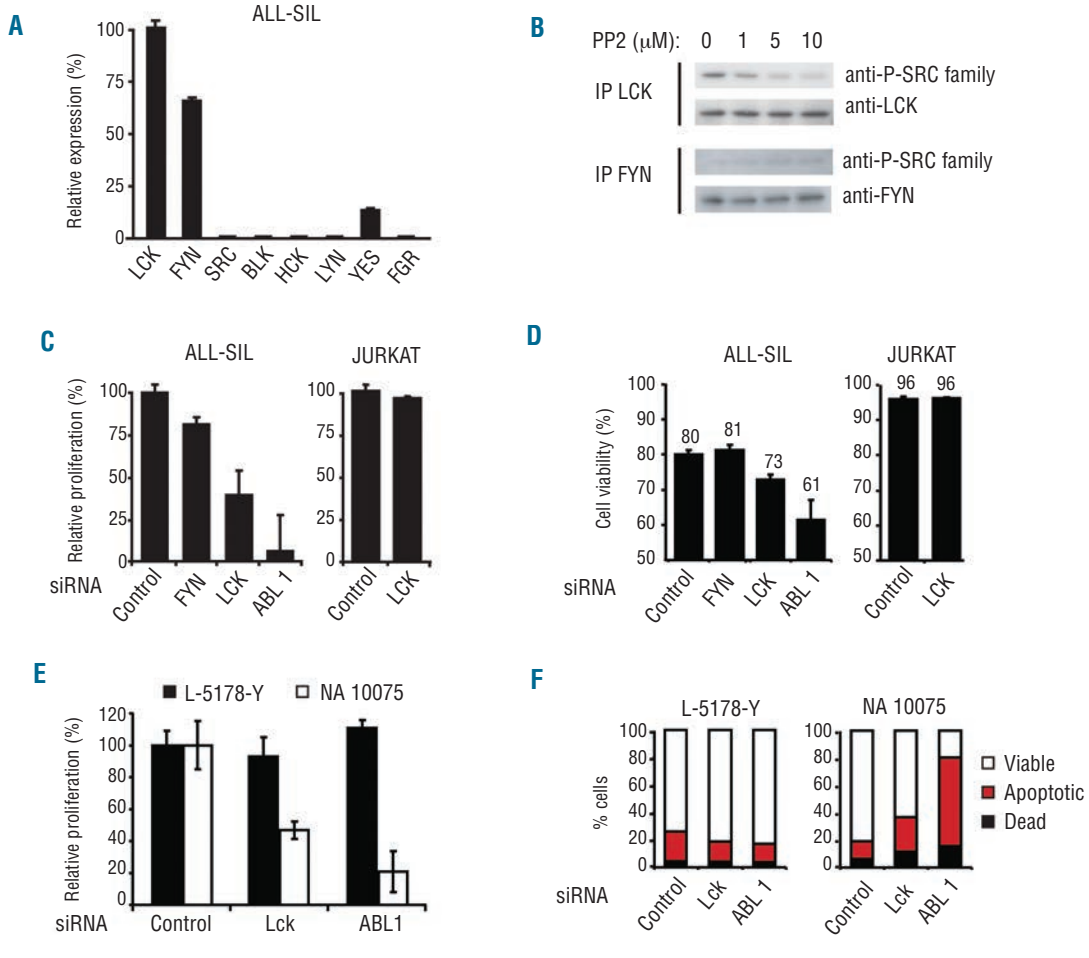

Figure 2. LCK is critical for NUP214-ABL1-positive T-ALL cell lines. (A) qRT-PCR analysis of SRC family kinase expression in ALL-SIL cells. (B) Immunoprecipitation (IP) of LCK or FYN from ALL-SIL cell lysates. Cells were pretreated with indicated concentrations of PP2 inhibitor. Phosphorylation of the precipitated proteins was assessed with an anti-phospho-SRC family kinase (Tyr416) antibody. (C) Proliferation of NUP214-ABL1-positive (ALL-SIL) and NUP214-ABL1-negative (JURKAT) cells after electroporation with the indicated siRNA. The average ±SD of triplicates is shown. (D) Percentages of viable cells after electroporation with the indicated siRNA. (E) Proliferation of NUP214-ABL1-positive (NA10075) and NUP214-ABL1-negative (NA10073) cells after electroporation with the indicated siRNA. The average ±SD of triplicates is shown. (F) Percentages of viable cells after electroporation with the indicated siRNA. siRNA knock-down efficiencies are displayed in *Online Supplementary Figures S1 and S2*.

ly developed to identify specific interactors of BCR-ABL1.[16] Notably, the nine selected candidate NUP214-ABL1 interactors were very rarely observed when screened against an extensive internally curated interactor database. This database has been generated from numerous interaction proteomics experiments performed with hundreds of different bait proteins from a range of different cell lines. Our findings imply that these particular nine proteins are specific interactors of NUP214-ABL1. Interestingly, the list of nine candidate NUP214-ABL1 interacting proteins did not show any overlap with the BCR-ABL1 interactors that were recently characterized using a similar experimental design,[16]

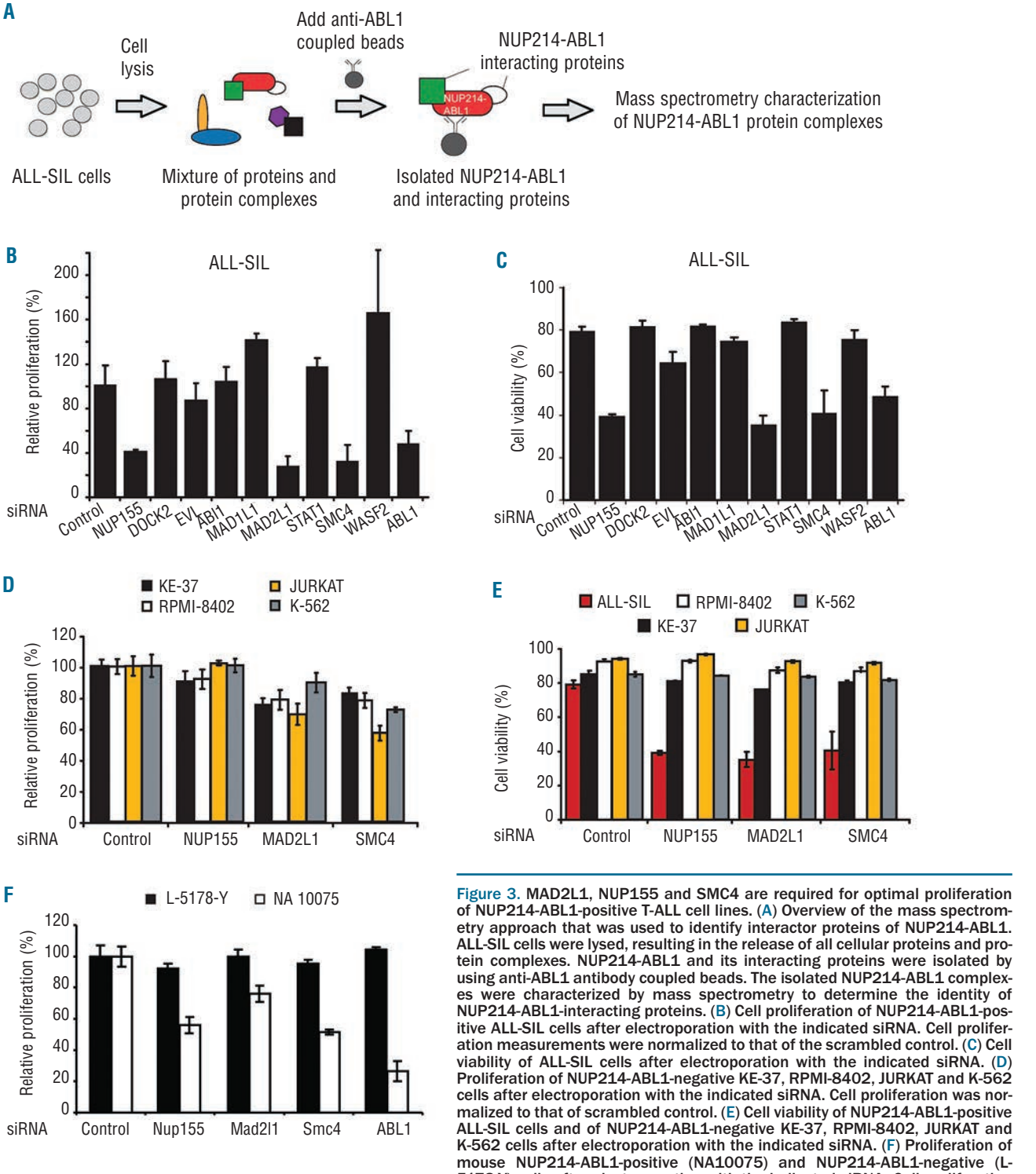

Figure 3. MAD2L1, NUP155 and SMC4 are required for optimal proliferation of NUP214-ABL1-positive T-ALL cell lines. (A) Overview of the mass spectrometry approach that was used to identify interactor proteins of NUP214-ABL1. ALL-SIL cells were lysed, resulting in the release of all cellular proteins and protein complexes. NUP214-ABL1 and its interacting proteins were isolated by using anti-ABL1 antibody coupled beads. The isolated NUP214-ABL1 complexes were characterized by mass spectrometry to determine the identity of NUP214-ABL1-interacting proteins. (B) Cell proliferation of NUP214-ABL1-positive ALL-SIL cells after electroporation with the indicated siRNA. Cell proliferation measurements were normalized to that of the scrambled control. (C) Cell viability of ALL-SIL cells after electroporation with the indicated siRNA. (D) Proliferation of NUP214-ABL1-negative KE-37, RPMI-8402, JURKAT and K-562 cells after electroporation with the indicated siRNA. Cell proliferation was normalized to that of scrambled control. (E) Cell viability of NUP214-ABL1-positive ALL-SIL cells and of NUP214-ABL1-negative KE-37, RPMI-8402, JURKAT and K-562 cells after electroporation with the indicated siRNA. (F) Proliferation of mouse NUP214-ABL1-positive (NA10075) and NUP214-ABL1-negative (L-5178-Y) cells after electroporation with the indicated siRNA. Cell proliferation was normalized to that of scrambled control. The average ±SD of three repeats is shown for all siRNA knock-down experiments. siRNA knock-down efficiencies are displayed in *Online Supplementary Figure S3*.

further decreasing the likelihood that the identified proteins were interacting with endogenous ABL1, which was inevitably co-immunoprecipitated with NUP214-ABL1 in this experimental approach.

To investigate whether any of the nine NUP214-ABL1 interactors are required for proliferation and/or viability of NUP214-ABL1-positive cells, we performed siRNA knock-down of each of these proteins in ALL-SIL cells. Knock-down of DOCK2, ABI1, MAD1L1, STAT1 or WASF2 did not significantly reduce the proliferation or viability of ALL-SIL cells. In contrast, knock-down of NUP155, MAD2L1 and SMC4 strongly inhibited proliferation and survival as compared to the levels in cells treated with scrambled control siRNA (Figure 3B,C). Minor effects were observed upon EVL knock-down. To distinguish NUP214-ABL1-specific effects from a general requirement of these proteins for cellular proliferation and survival, we also knocked-down NUP155, MAD2L1 and SMC4 in three T-ALL cell lines that do not express NUP214-ABL1 (KE-37, JURKAT and RPMI-8402), as well as in a BCR-ABL1-positive CML cell line (K-562). Knock-down of NUP155 did not significantly affect the proliferation of these control cell lines, whereas knock-down of MAD2L1 or SMC4 did cause minor effects on cell proliferation, albeit much less pronounced than in the ALL-SIL cells (Figure 3D). Moreover, whereas the viability of ALL-SIL cells was drastically reduced by knock-down of NUP155, MAD2L1 and SMC4, the cell viability of the NUP214-ABL1-negative lines was unaffected by knock-down of each of these proteins (Figure 3E). We also knocked-down MAD2L1, NUP155, or SMC4 in mouse T-cell leukemia cell lines, using an independent set of mouse siRNAs. In agreement with the data obtained in the human cell lines, knock-down of these interaction partners specifically reduced the proliferation of the NUP214-ABL1-positive cell line NA10075, whereas these effects were not observed in the NUP214-ABL1-negative cell line L-5178-Y (Figure 3F). Taken together, our results indicate that the proliferation and survival of NUP214-ABL1-positive cells are dependent on MAD2L1, NUP155 and SMC4.

Next, we tried to confirm binding of MAD2L1, NUP155, and SMC4 to NUP214-ABL1 in independent co-immunoprecipitation experiments. We could co-immunoprecipitate endogenous NUP214-ABL1 with endogenous MAD2L1 in NUP214-ABL1-positive ALL-SIL cells. This interaction was absent in NUP214-ABL1-negative JURKAT cells or BCR-ABL1-positive K-562 cells, indicating a specific interaction of NUP214-ABL1 with MAD2L1 (Figure 4A). We were, however, unable to confirm interactions with endogenous NUP155 or SMC4 due to technical limitations concerning the antibodies that were available for these interacting proteins. To circumvent these limitations, further interaction studies were performed in HEK293T cells, in which we expressed NUP214-ABL1 in combination with HA-tagged NUP155 or SMC4. Under these conditions, we were able to co-immunoprecipitate NUP155 with NUP214-ABL1 (Figure 4B). Of note, we also detected a very weak interaction with BCR-ABL1. However, taking into account that BCR-ABL1 was immunoprecipitated in much larger quantities than NUP214-ABL1, our data indicate a specific interaction between NUP214-ABL1 and NUP155. An interaction between SMC4 and NUP214-ABL1 could not be detected by co-immunoprecipitation. However, in immunofluorescence experiments, we observed that expression of NUP214-ABL1 in HEK293T cells causes a redistribution of endogenous SMC4 from diffuse cytoplasmic and nuclear staining in control cells towards co-localization at the nuclear envelope upon expression of NUP214-ABL1, strongly pointing to an, at least indirect, interaction between NUP214-ABL1 and SMC4 (Figure 4C).

Discussion

The discovery of the ABL1 kinase inhibitor imatinib, the first successful example of molecularly tailored therapy, has revolutionized the treatment of BCR-ABL1-positive CML and B-ALL, as well as of other tumors that depend on imatinib-sensitive tyrosine kinases.[20] It is now well established that the oncogenic NUP214-ABL1 fusion kinase is also sensitive to imatinib and that proliferation of cell lines expressing NUP214-ABL1 is inhibited by imatinib.[4,6,8] However, we still await more clinical experience to evaluate the therapeutic potential of imatinib in NUP214-ABL1-positive T-ALL. Because of the low number of patients carrying the NUP214-ABL1 fusion, reports on the clinical responses of such patients are limited so far. In human T-ALL patients, NUP214-ABL1 invariably shows intra- or extra-chromosomal amplification, with as many as 20-30 copies per cell.[4,21] As over-expression of BCR-ABL1 is a known mechanism of imatinib resistance,[22-25] we predict that this amplification of NUP214-ABL1 may contribute to imatinib resistance in NUP214-ABL1-positive T-ALL. Another well-known mechanism of resistance to imatinib in BCR-ABL1-positive leukemias is the emergence of resistance due to point mutations,[9] a phenomenon that we also expect in NUP214-ABL1-positive T-ALL. In this study, we therefore aimed to identify proteins in the signaling and interaction network of NUP214-ABL1 that are critical for the survival and proliferation of T-ALL cells, as these proteins might serve as alternative drug targets in imatinib-resistant NUP214-ABL1 positive T-ALL.

BCR-ABL1 activates the SRC family kinases LYN, FGR and HCK in pre-B-cells and these kinases are required for B-ALL induction by BCR-ABL1 in a mouse model.[15] Moreover, imatinib-resistant BCR-ABL1-positive CML blast crisis cells can be forced into apoptosis by targeting LYN.[14] Based on these results, we hypothesized that SRC family kinases may also play an important role in NUP214-ABL1-mediated transformation. Indeed, we found that NUP214-ABL1 positive human and mouse cell lines are sen-

Table 1. List of selected potential NUP214-ABL1 interactors. The column 'peptides' refers to the number of unique peptides that were identified for that corresponding protein in the mass spectrometry analysis. 'Coverage' refers to the percentage of sequence of the total protein that was identified in our mass spectrometry analysis.

Gene	Full Gene Name	Peptides	Coverage (%)
NUP155	Nucleoporin 155 kDa	33	30.7
DOCK2	Dedicator Of Cytokinesis 2	3	1.7
EVL	Enah/Vasp-like	5	14.4
ABI1	ABL-Interactor 1	3	8.5
MAD1L1	Mitotic Arrest Deficient 1-Like 1 (yeast)	10	16.7
MAD2L1	Mitotic Arrest Deficient 2-Like 1 (yeast)	2	8.8
STAT1	Signal Transducer and Activator of Transcription 1	4	6.6
SMC4	Structural Maintenance of Chromosomes 4	4	3.8
WASF2	WAS protein Family, member 2	3	6.6

sitive to the SRC family kinase inhibitor PP2, an effect which is primarily mediated through inhibition of LCK. LCK is a central kinase in T-cell precursors for the transition of CD4/CD8 double negative to double positive thymocytes and stimulates mitosis of early T-cell precursors.[26] Moreover, mice transgenic for wild- type or constitutively active Lck develop thymic tumors and rare T-ALL cases have been described with overexpression of LCK by t(1;7)(p34;q34) juxtaposing *LCK* to the strong promoter sequences of the *TRB@* locus.[27,28] These data, together with our finding of required LCK activity for proliferation of NUP214-ABL1-transformed cells, establish LCK as an important drug target in the pathogenesis of T-ALL.

Our finding that LCK is required for NUP214-ABL1 in T-ALL has clinical implications. The Food and Drug Administration-approved multi-kinase inhibitors dasatinib and bosutinib inhibit both ABL and SRC kinases and are used for the treatment of imatinib-resistant and -sensitive BCR-ABL-positive malignancies.[29,30] Interestingly, both drugs very potently inhibit LCK activity *in vitro* with IC_{50} values of ~1-2 nM.[31,32] We showed that NUP214-ABL1 activity is inhibited by dasatinib in *in vitro* kinase assays, that proliferation of NUP214-ABL1-positive cells is inhibited by dasatinib and that dasatinib inhibits NUP214-ABL1-positive leukemogenesis in mouse xenografts and primary NUP214-ABL1-positive T-ALL lymphoblasts.[6,9] Furthermore, dasatinib and bosutinib have much narrower spectra of point mutations that cause drug resistance as compared to imatinib.[33] The clinical potential of dasatinib or bosutinib for the treatment of NUP214-ABL1-positive T-ALL is further supported by a case report showing induction of rapid complete hematologic and cytogenetic remission after upfront dasatinib monotherapy in a patient with a NUP214-ABL1-positive T-ALL.[34] Based on this range of pre-clinical and emerging clinical data, one may prefer dasatinib over imatinib in NUP214-ABL1-positive T-ALL patients. Ideally, the efficacy of dual SRC-ABL1 inhibitors *versus* ABL1 inhibitors should now be compared in experiments with primary NUP214-ABL1-positive leukemia cells.

We previously described that NUP214-ABL1 and BCR-ABL1, despite carrying the same portion of the ABL1 kinase, differ in almost any biological property that we have studied, such as subcellular localization, mechanism of initiation of kinase activity, phosphorylation pattern, enzymatic activity, kinase inhibitor sensitivity and substrate spectrum.[6,7] Analysis of the proteins interacting with NUP214-ABL1 in this work again indicates a strong difference from BCR-ABL1. None of the core interaction partners that we identified for BCR-ABL1 (GRB2, SHC1, CRK-I, CBL, p85, STS-1, and SHIP-2) was identified in the mass spectrometric analysis of the NUP214-ABL1 protein complexes.[16] This dramatically different composition of NUP214-ABL1 and BCR-ABL1 complexes might be the result of a combination of the T-cell *versus* granulocyte/B-cell context in which NUP214-

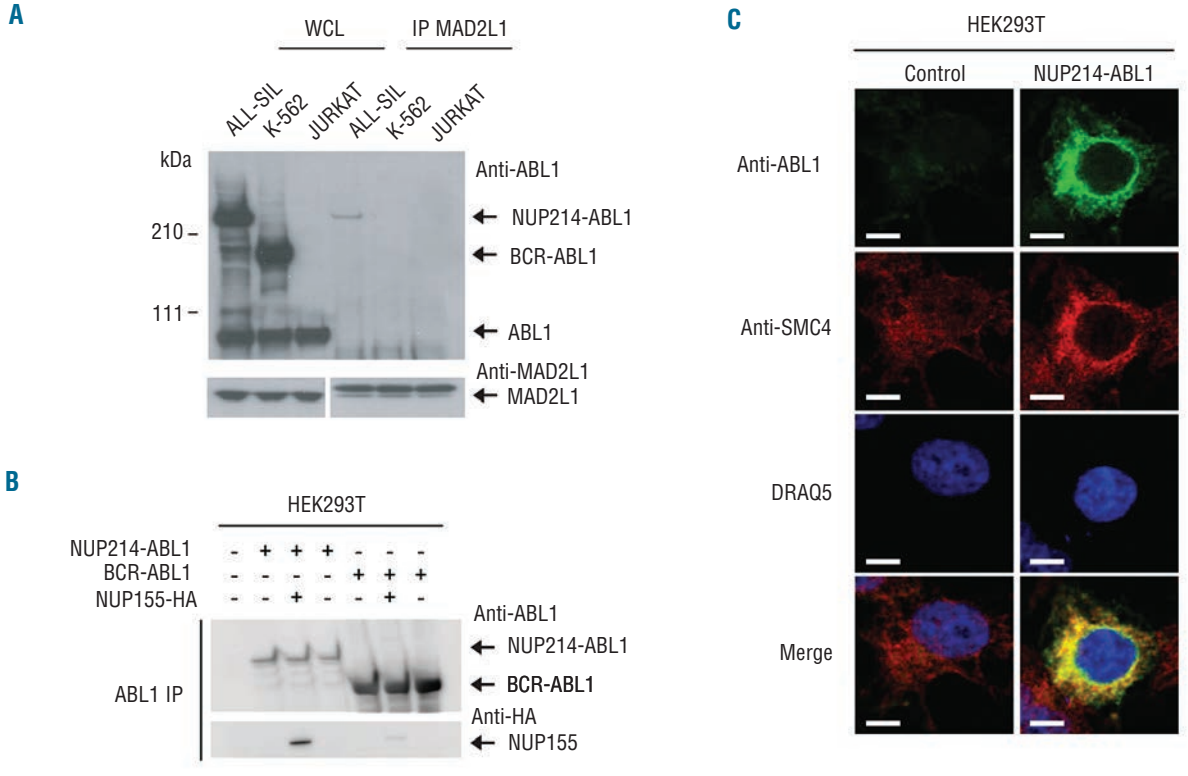

Figure 4. MAD2L1, NUP155 and SMC4 interact or co-localize with NUP214-ABL1. (A) Endogenous MAD2L1 protein was immunoprecipitated (IP) from ALL-SIL (NUP214-ABL1-positive), K-562 (BCR-ABL1-positive) and JURKAT cells (no ABL1 fusion) with anti-MAD2L1 antibody. Co-immunoprecipitation of NUP214-ABL1 or BCR-ABL1 was assessed on the shown western blot with anti-ABL1 antibody. (WCL: whole cell lysate) (B) ABL1 protein was immunoprecipitated from HEK293T cells that were transfected with the indicated combinations of NUP155-HA and ABL1 fusion constructs. Co-precipitation of HA-tagged NUP155 was assessed with anti-HA antibody. (C) Subcellular localization of NUP214-ABL1, ABL1 and SMC4 was investigated by immunofluorescence in HEK293T cells. Control HEK293T cells display low endogenous levels of ABL1 and diffuse SMC4 staining. In contrast, ABL1 and SMC4 co-localize at the nuclear pore in transfected cells expressing NUP214-ABL1. DRAQ5 stains DNA and visualizes the nucleus. Scale bar: 5 μm.

ABL1 and BCR-ABL1 occur, conformational differences of the ABL1 portion of the two fusion oncoproteins and differences in their subcellular localizations. NUP214-ABL1 resides partially at the cytoplasmic side of the nuclear envelope and in the cytoplasm whereas BCR-ABL1 is localized strictly to the cytoplasm.[7] The mass spectrometric studies on the BCR-ABL1 protein complexes[16] were performed under the same conditions and in the same laboratory as the NUP214-ABL1 complexes. It can, therefore, be excluded that the observed differences between BCR-ABL1 and NUP214-ABL1 are due to different immunoprecipitation or mass spectrometry conditions. We also confirmed that BCR-ABL1 core interactors were expressed in NUP214-ABL1-positive cells and *vice versa*, thereby excluding that the absence of interaction of BCR-ABL1 interactors with NUP214-ABL1 is caused by a lack of expression of these proteins in NUP214-ABL1-positive cells and *vice versa*.

In this study, we identified NUP155 as an interactor of NUP214-ABL1 and knock-down of NUP155 reduced proliferation of NUP214-ABL1-positive cells. These data fit within our previous observations that NUP214-ABL1 interacts with other nucleoporins such as NUP62, NUP88 and RANBP2 (= NUP358) and that NUP214-ABL1 depends on interaction with these nucleoporins for its activity.[7] In contrast to NUP62, NUP88 and RANBP2, no direct interactions between NUP214 and NUP155 have been described. However, our data indicate that in the context of NUP214-ABL1, NUP155 interacts with NUP214 (directly or indirectly) in the nuclear pore complex.

In addition to NUP155, SMC4, a member of the condensing complex converting interphase chromatin into condense chromosomes, and MAD2L1, a spindle checkpoint regulator protecting cells from abnormal chromosome segregation, were also detected in NUP214-ABL1 complexes and were required for proliferation of NUP214-ABL1-positive cells. It remains to be determined whether NUP155, SMC4 and MAD2L1 are substrates phosphorylated by NUP214-ABL1 and, if so, whether the function of these proteins is affected in NUP214-ABL1-positive cells. Preliminary experiments failed to detect NUP214-ABL1-dependent tyrosine phosphorylation of these three proteins (*Online Supplementary Figure S4*). Another mechanism by which NUP214-ABL1 could affect the function of these proteins is by altering their subcellular localization. Indeed, for SMC4 we observed a clear change in localization of the cellular SMC4 pool towards the nuclear envelope. It will be interesting to test how this affects SMC4 function. Based on the role of SMC4 and MAD2L1 in cellular processes such as chromosome condensation and spindle checkpoint regulation, it is not unlikely that altered function of these proteins promotes transformation of cells by NUP214-ABL1.

It is worth noting that in our interaction proteomic studies, we were able to confirm known interactions of NUP214-ABL1 with NUP88 and PTPN2.[7,35] PTPN2 is a phosphatase that we previously found to be deleted in T-ALL and which we showed exerts a negative regulatory effect on NUP214-ABL1 tyrosine kinase activity.[35] Interestingly, we also identified STAT1 as a member of NUP214-ABL1 protein complexes. Endogenous NUP214 is known to import STAT1 in the nucleus under normal, steady state conditions.[36] Our knock-down studies, however, suggest that NUP214-ABL1-positive cells do not depend on STAT1 for their survival.

As mentioned earlier, the NUP214-ABL1 fusion was recently described to occur also in B-ALL patients.[5,37] At this moment it remains to be determined to what extent NUP214-ABL1 in T-ALL and B-ALL contexts resemble each other and whether the findings we describe above in the context of T-ALL cells could also be applicable to B-ALL.

NUP214-ABL1 usually presents with episomal amplification such that the number of copies varies considerably from cell to cell in the same patient.[4,21] In some patients, it even occurs as a secondary change not seen in all cells. Therefore, to obtain durable therapeutic responses in NUP214-ABL1-positive T-ALL, we anticipate that combinations of agents hitting NUP214-ABL1 and/or the proteins on which NUP214-ABL1 relies, together with other targeted agents and/or low doses of chemotherapy will be required.

In conclusion, we identify LCK, MAD2L1, SMC4 and NUP155 as proteins on which NUP214-ABL1-positive T-ALL tumor cells depend critically for their proliferation, identifying these proteins as potential drug targets in NUP214-ABL1-positive T-ALL. Targeting LCK in NUP214-ABL1 could easily be addressed in the clinical treatment schemes of NUP214-ABL1-positive T-ALL patients, given the availability of dasatinib and bosutinib co-targeting ABL1 and LCK. Our work thus provides a molecular rationale for testing dasatinib and bosutinib alone or in combination with other targeted agents and/or chemotherapy in patients with NUP214-ABL1-positive T-ALL.

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